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Enzyme Immunoassays for Viral Diseases

D. E. Bidwell, Ann Bartlett, and A. Voller

From the Nuffield Institute of Comparative Medicine, The Zoological Society of London, London, England

Microplate enzyme-linked immunosorbent assays (ELISA) for viral diseases were investigated with special reference to rubella. Standardization of the carrier plates, antigens, conjugates, and substrate was found to be essential. The ELISA results were compared with results of hemagglutination-inhibition tests for rubella, and ELISA was used in an epidemiologic study. Antibodies to cytomegalovirus, measles, adenovirus, coxsackieviruses, and herpesviruses were also assayed by ELISA. ELISA has veterinary applications in detection of respiratory syncytial virus and Newcastle disease virus, and it has been used for assay of plant viruses.

The diagnosis of viral diseases presents some special problems because direct demonstration of the organism may only be possible by slow and expensive methods, for example, tissue culture and animal inoculation. More recently, immunofluorescence and immunoenzymatic staining of the organisms have been used, but serology is still the mainstay of the diagnostic laboratory. A bewildering array of serologic tests are in use, and a sensitive test with wide applicability is needed. We feel that the enzyme-linked immunosorbent assay (ELISA) is a promising candidate for this purpose.

The ELISA as outlined by Engvall and Perlmann [1]is deceptively simple. It is easy to set up on a single occasion, and it is extremely sensitive. However, our experiences over the last two years have shown that many factors can affect the outcome of the test, and these factors must be rigidly controlled if the test is to be used in the viral diagnostic laboratory. This report deals with some of these variables, with special reference to the rubella system.

Materials and Methods

We have used the microplate modification [2] of the ELISA method of Engvall and Perlmann [1], which was performed as follows. The wells of microhemagglutination plates were sensitized by addition of 0.2 or 0.3 ml of rubella antigen (no. 30-954; Microbiological Associates, Bethesda, Md.) diluted in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6, containing 0.02% sodium azide). After incubation in a humid chamber, the plates were washed and the next reagent was added.

Washing. The wells were emptied and refilled with 0.15 M phosphate-buffered saline containing 0.05% Tween-20. This procedure was repeated three times at 3-min intervals. The plates were then shaken dry, and the next reagent was added immediately. The diluted test sera (0.2 or 0.3 ml) were added to separate wells, and the plate was kept in a humid chamber. Plates were then washed as before. Diluted antiserum to human globulin (0.2 or 0.3 ml) labeled with alkaline phosphatase was added to each well; the plates were kept in a humid chamber and then washed again. Next, 0.2 or 0.3 ml of the enzyme substrate solution (*p*-nitrophenyl phosphate, 1 mg/ml in 10% diethanolamine buffer, pH 9.8) was added to each well. After a suitable time 50 μl of 3 M NaOH was added to each well to stop the enzyme reaction. The contents of each well were then removed, and the absorbance at 405 nm was determined in a spectrophotometer. Samples were diluted if readings were above the accurate range of the spectrophotometer.

Results and Discussion

The first factor of crucial importance to the assay is the type of carrier plate used. We set up identical tests for rubella antibody (using positive and negative reference sera) on polystyrene plates (M29AR), polyvinyl plates (220-29), and polystyrene plates specially treated for tissue cul-

Please address requests for reprints to Dr. A. Voller at his present address: London School of Hygiene and Tropical Medicine, Keppel Street, London WC1, England.

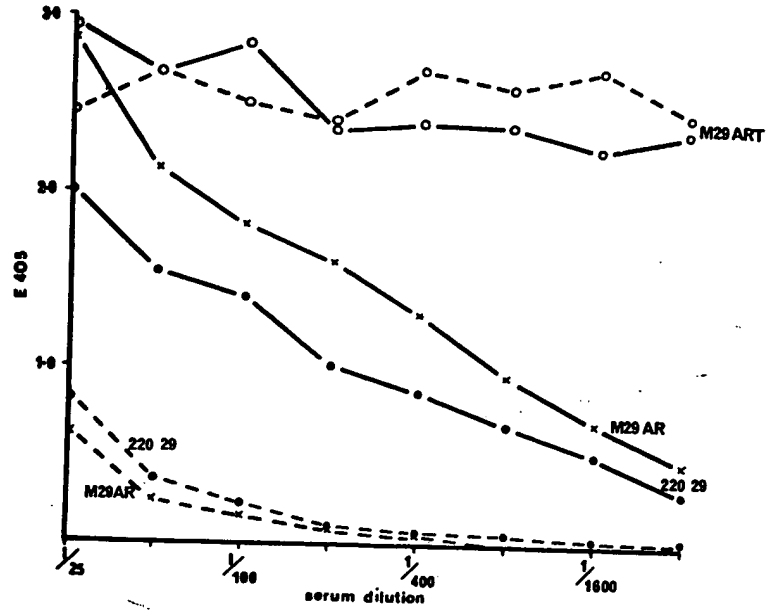

Figure 1. Results of the microplate enzyme-linked immunosorbent assay for rubella on different types of carrier plates: M29ART = polystyrene tissue culture; M29AR = polystyrene; 22029 = polyvinyl chloride. Solid lines represent positive sera; dotted lines represent negative sera. Absorbance at 405 nm (E 405) is noted on the ordinate.

ture (M29ART) all from Dynatech Laboratories, Alexandria, Va. The results are shown in figure 1. Although polystyrene and polyvinyl plates gave rather similar results, allowing good discrimination between the two reference sera, the tissue culture plates were quite useless. The latter had a high nonspecific uptake that masked any differences between the positive and negative sera. It is essential to evaluate the plates for every new system, as one cannot extrapolate from one system to another. We have found that the polyvinyl plates are better for coating with immunoglobulins, for antigen detection, and for use in tests for antibodies to some protozoa. There were even variations between different batches of the same type of plates, but now that the manufacturers are aware of the stringent requirements for ELISA plates, particular care will be taken with them. Previously, for other serologic tests it has been the physical configuration of the wells in the plates that was important, but it is now recognized that for ELISA the plates' chemical composition is also relevant.

The antigen coating of the plates is also critical, and the optimal conditions can be most easily determined by checkerboard titrations of dilutions of the antigen against the positive and negative reference sera. Such a titration is illustrated in table 1. We normally aim to determine the most economical dilution of antigen that gives a reading of ≥ 1.0 with the positive serum and <0.2 with the negative serum. In the series illustrated these requirements were obtained with an antigen dilution of 1:200 and a serum dilution of 1:100 or 1:200. It can be useful to carry out similar tests on a "negative antigen." In the case of viruses, the overlay or extract from uninfected tissue culture cells is usually used. For reasons that we do not understand, occasionally sera from patients with high levels of rubella antibody also react with the control antigen. This reaction can be eliminated by dilution of the test sera in phosphate-buffered saline and Tween containing dilute negative antigen.

There are considerable differences between the viral antigens produced by different laborator-

Table 1. Effect of various dilutions of antigen and test serum on the results of microplate enzyme-linked immunosorbent assays for antibody to rubella.

Dilutions of rubella antigen	Results at dilutions of positive reference serum				
	1:100	1:200	1:400	1:800	1:1,600
1:100	1.52	1.28	1.02	0.69	0.50
1:200	1.33	1.14	0.78	0.71	0.46
1:400	1.16	0.96	0.79	0.55	0.44
1:800	1.1	0.92	0.77	0.50	0.32
1:1,600	1.0	0.86	0.86	0.50	0.30

NOTE. Data are given as absorbance at 405 nm after 45 min.

ies. Some commercially produced rubella antigens have no activity whatsoever in the ELISA system. Because of the variability between individual batches of antigen, it is necessary to titrate each new lot. There is a need for reference antigenic materials against which others can be standardized. Alternatively, activity of antigens could be expressed in terms of results in ELISA tests carried out under defined conditions against reference sera. Many of the latter are already available as national and international standards.

Coating of the plastic with the antigen is done by passive adsorption and (in the case of viruses) occurs rapidly. We were unable to detect any differences in the rubella system between antigen coating for 30 min at 4 C and for 18 hr at 4 C. The optimal conditions for each new system are best determined in pilot experiments. For convenience we usually use the sensitized plates immediately. However, after the antigen solution is emptied the plates can be dried or lyophilized. They retain their full activity for at least several weeks.

As mentioned above, the optimal dilution of test serum is found initially by checkerboard titration. In the subsequent routine tests, sera may be tested at one dilution to get good discrimination between weak positive and negative results and at another dilution to separate different high positive results. The optimal incubation time for serum must also be determined by trial and error for each new system. In practice in the microplate ELISA for viral antibody, there is usually little difference between 2-hr and 18-hr incubations.

The conjugate is another critical component in the ELISA tests. Each new batch must be standardized against the relevant immunoglobulin; then the working dilution must be determined in the system under investigation. This is best done by checkerboard titrations of the conjugate with the positive and negative reference sera. The biggest differences between positive and negative sera are obtained with strong conjugates incubated for a long time. However, in the interests of economy of materials and time, a compromise that gives adequate separation of positive and negative results is used. This compromise involves a choice between a rapid result with a more expensive test by the use of strong conjugates for a short time, or a cheaper but more time-consuming test by use of more dilute conjugates with a longer incubation time. The effects of varying these factors in the rubella system are shown in figure 2.

The final step, substrate incubation, is also critical. The objective is to measure the reaction rate, and if the expensive apparatus for this procedure is available, this rate can be determined at any time before the substrate is exhausted. Because most diagnostic laboratories have less demanding requirements and budget restrictions, the amount of substrate degradation after a given time is the final measurement. Simple spectrophotometers can be used for this purpose. Under these conditions, the enzyme breakdown of the substrate is stopped after an interval; this interval can be constant if all other conditions of the test, including temperature and time, are held constant. In practice this constancy is difficult to achieve, and it is easier to control the final substrate reaction by the inclusion of samples of reference serum on each plate. A number of wells containing the reference serum are monitored in the final stage to determine the rate of substrate degradation [3]. When the reading of the reference sample reaches a predetermined figure, the

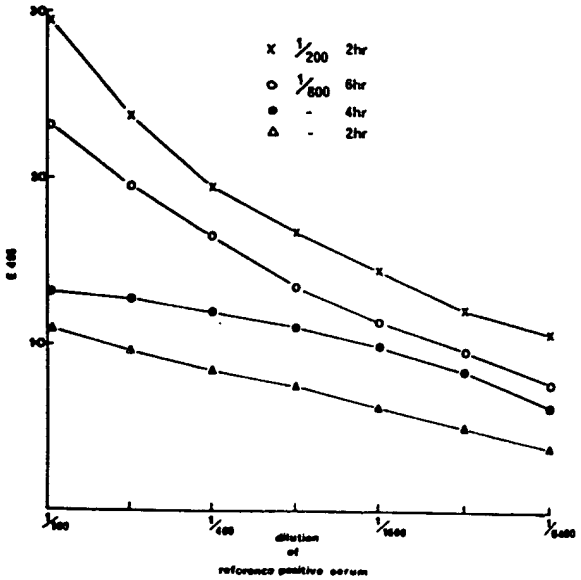

Figure 2. Results of microplate enzyme-linked immunosorbent assay for antibody to rubella, with different conditions for incubation of conjugate. Absorbance at 405 nm (E 405) is noted on the ordinate.

Table 2. Results of the microplate enzyme-linked immunosorbent assay (ELISA) for rubella, in terms of titers of HAI antibody.

Reciprocal HAI titers (no. tested)	ELISA results*		
	No. negative	No. positive	Mean absorbance
<8 (31)	26	5	0.15
16–32 (20)	2	18	0.34
64–128 (72)	0	72	0.40
256–512 (49)	0	49	0.49
>1,024 (16)	0	16	0.71

NOTE. Data are from [4].

*Mean absorbance was measured at 400 nm; the result was negative if absorbance was <0.2, and positive if absorbance was ≥0.2.

reactions in all other test samples are stopped, and results are read. We usually aim for an absorbance at 405 nm of 1.0 with the positive reference, with substrate incubated for 15–60 min. Shorter times lead to errors due to timing, and longer intervals become inconvenient. The inclusion of reference samples also compensates for variations in other stages of the test.

Applications of the test. The first reported use of ELISA in virology was for the detection and measurement of rubella antibody [4]. It was found that the overall positivity rate of the ELISA and HAI tests correlated well and that increasing HAI titers were reflected by higher ELISA values (table 2). In a later study [5] we reported on the use of enzyme conjugates of antibody to IgM for the detection of the primary antibody response in vaccinated people. This use of class-specific conjugates in detection of recent rubella infection would be of considerable value in the clinical context. The work on rubella has now been extended to include study of a small population in New Guinea (table 3). Because of the sensitivity of the ELISA, it was possible to perform the test even on the small amounts of blood collected in capillary tubes by pricking of the finger. The results reflect those of other serologic surveys in similar areas. The epidemiologic potential is further emphasized by the fact that a number of other ELISA tests were also performed on those same small samples of plasma. The results of the ELISA assays for measles antibody are also shown in table 3. Previously, Voller and Bidwell [5] were able to differentiate on the basis of ELISA values between the people with recent experience with measles and those without any history of recent infection.

Antibody to cytomegalovirus has also been assayed by ELISA [5, 6]. The ELISA for cytomegalovirus used much less antigen than the routine CF test. This characteristic could be important because the antigen is rather difficult to produce.

There have been few other reports to date on the use of ELISA on clinical samples, but Voller et al. [7] did show that ELISA tests of paired sera from patients suspected of Japanese B encephalitis could be useful: all of the convalescent samples had higher values than those taken in the acute phase.

In feasibility studies on the use of various com-

Table 3. Results of the microplate enzyme-linked immunosorbent assay for antibody to rubella and measles in sera of subjects in New Guinea.

Age in years (no. tested)	Rubella		Measles	
	Positive (%)	Absorbance (mean ± SE)	Positive (%)	Absorbance (mean ± SE)
<2 (18)	5.5	0.08 ± 0.01	11	0.15 ± 0.06
2–4 (41)	49	0.28 ± 0.03	44	0.38 ± 0.08
5–9 (78)	74	0.45 ± 0.03	92	0.63 ± 0.04
10–16 (63)	90	0.58 ± 0.03	100	0.67 ± 0.04
17–30 (65)	94	0.61 ± 0.03	97	0.55 ± 0.02
>30 (61)	99	0.61 ± 0.03	95	0.52 ± 0.03

NOTE. Polystyrene plates were coated for 18 hr at 4 C with rubella antigen diluted 1:200 (no. 30-954; Microbiological Associates, Bethesda, Md.) or measles antigen diluted 1:100 (no. 30-850; Microbiological Associates). Test sera diluted 1:100 were incubated for 2 hr at room temperature. The conjugate, alkaline phosphatase-labeled antiserum to human immunoglobulin diluted 1:1,000, was incubated for 2 hr. Duration of substrate incubation was about 60 min but depended on the reference serum. Sera were considered positive if absorbance at 405 nm was ≥0.2.

Table 4. Results of the microplate enzyme-linked immunosorbent assay for plum pox virus in plant extracts.

Condition of plants	Absorbance at dilutions of plant extract				
	1:10	1:50	1:250	1:1,250	1:6,250
Infected with plum pox virus	6.9	3.7	1.8	0.7	0.3
Infected with hop mosaic virus	0.2	0.15	0.12	0.13	0.12
Healthy	0.16	0.13	0.12	0.11	0.14

NOTE. Polyvinyl plates were coated for 5 hr at 37 C with IgG fractions (10 μg/ml) of antiserum to plum pox virus. Plant extracts in phosphate-buffered saline and Tween were incubated overnight at 4 C. Conjugate, alkaline phosphatase-labeled antiserum to plum pox virus, at a dilution of 1:750, was incubated for 4 hr at 25 C. Substrate was incubated for 15 min. Absorbance at 405 nm was measured after 15 min.

mercial viral antigens in ELISA tests, Voller et al. [7] obtained successful results with adenovirus, coxsackievirus, and herpesvirus. In each system it was possible to differentiate easily between the positive and negative reference sera. As yet the test has not been used on clinical samples in these diseases. Dr. Blaudin de The, in collaboration with our laboratory, has performed preliminary ELISA tests for antibody to Epstein-Barr virus and has obtained promising results, in that high values were found for patients with Burkitt's lymphoma and lower values for those with infectious mononucleosis.

The ELISA also has great potential in veterinary viral diseases. With Dr. James Stott we have set up tests for antibody to respiratory syncytial virus. We obtained high values with sera from germ-free cattle with induced infections with respiratory syncytial virus, and there were no cross-reactions with sera from similar cattle with other induced viral infections. We have also performed preliminary trials with Dr. George Parsons using ELISA for detecting antibody to Newcastle disease virus in chickens. There was good correlation between mean ELISA values and HAI titers.

Our discussion thus far has been devoted to tests for antibody. However the double antibody sandwich ELISA method can be used to assay the actual virus by means of plates coated with virus-specific antibody and a conjugate of enzyme-labeled virus-specific antibody.

With Dr. Michael Clark we have used this method to assay plant viruses [8] and have found it to be extremely sensitive and specific (table 4). The same method could be applied to the assay of viruses in humans, animals, and tissue culture.

This survey of the current state of ELISA in viral diseases can only serve as a pointer to the many other applications that we anticipate in the near future.

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